

"A Preliminary Note on the Morphology and Distribution of the Organism found in the Tsetse Fly Disease." By H. G. PLIMMER and J. ROSE BRADFORD, F.R.S., Professor Superintendent of the Brown Institution. Received June 12,— Read June 15, 1899.

(From the Laboratory of the Brown Institution.)

These observations are the result of an inquiry entrusted to us by the Tsetse Fly Committee of the Royal Society, at a meeting of the Committee on March 16, 1899.

The material for our investigations was obtained in the first place from a dog and a rat, inoculated with the blood of a dog suffering from the disease, by Mr. H. E. Durham, at Cambridge.

The organism found in the Tsetse Fly disease was discovered by Major Bruce, R.A.M.C., F.R.S., and was classed by him as a Trypanosoma. These belong to the order Flagellata, and, according to Bütschli, to the sub-group Monadina.

We will, in the first place, describe the adult form of the organism, such as is met with most frequently in the blood of a susceptible animal affected with the disease.

A. Description of the Adult Form of the Trypanosoma.

In freshly drawn blood examined as a hanging drop, or as a very thin layer in a cell, the adult form of the Trypanosoma can be easily studied. The latter method is the better, as the organism can be better seen and more accurately examined, in the thin, uniform layer of fluid than in the rounded drop. The easiest method of examining the blood in this way is to make, with a red-hot platinum loop and a small piece of paraffin, a thin ring of paraffin on an ordinary glass slide; the drop of blood is placed in the centre of the ring and a cover-glass placed on it, the thin layer of paraffin preventing pressure. If it be desired to keep the blood for continuous examination, it should be drawn into a graduated Pasteur pipette, and one-tenth part of a 5 per cent. solution of sodium citrate should be drawn up after it, then the blood and citrate solution should be carefully mixed in the bulb; the tube should then be sealed up, and drops can be taken from it as desired.

Under ordinary conditions of illumination the Trypanosoma, as seen in the blood, appears to consist of a uniform, homogeneous mass of protoplasm, of worm-like form, with at one end a thick, stiff extremity, and at the other a long, wavy flagellum. It is generally in active motion, and this is seen to be caused by the rapid lashing movement of the flagellum, and by the rapid contractions and relaxations of the

mass of protoplasm forming the body, and by the movements of an undulating membrane which is attached to one surface of the body, and which appears to undulate synchronously with the contractions of the protoplasmic body. This membrane is, excepting at the free edge, very transparent, and can be seen much better in citrated blood which has been thickened by the addition of a small drop of 1 per cent. gelatine solution, when its contour and attachments can be much better made out, owing to the slower rate of vibration effected by the thickened medium.

The general shape of the Trypanosoma, when rendered quiescent by this means but not killed, is that of a long oval, with one end blunt and the other continued into the flagellum ; the membrane is then seen to be attached to one side of the body ; it begins a little in front of the blunt end of the organism, and is continued at the end into the flagellum.

But with better illumination, such as a very oblique pencil of rays, or, better still, with monochromatic light (green or blue), the protoplasm is seen not to be homogeneous. The organism appears then as a highly refractive body, and near the middle, or between it and the flagellate end, is seen a large dark body much more refractive than the rest of the protoplasm ; this is the macronucleus. Near the thick, stiff end of the body a tiny still more refractive body (with monochromatic light nearly black) is seen, which is the micronucleus. The addition of a drop of 5 per cent. acetic acid makes both of these bodies much more distinct. At the stiff end of the Trypanosoma, in varying relation to the micronucleus, is seen a vacuole. There is no suggestion of a mouth or of any organs, but the protoplasm with the most careful illumination appears not to be uniform, which suggests an alveolar structure, as described by Bütschli. With the ordinary simple stains (haematoxylin, fuchsin, methylene-blue, thionin) the differentiation is not much better than can be observed by careful illumination of living unstained organisms, as these stains are with these, and similar organisms, too diffuse to be of any service. Acting on a method which Ehrlich originated in 1889, and which Romanowsky modified in 1891, and which has still been further elaborated by Ziemann in 1898, we have used a mixture of methylene-blue and erythrosin, which has enabled us to follow the different stages of the Trypanosoma with certainty. This method depends on the fact that when a basic and an acid stain are mixed together in certain proportions, a third neutral body is formed, which has a specific colour reaction with chromatin. By the use of this method we have been able to trace the various stages of the organism in the blood and organs of the affected animals, which is not possible with the ordinary stains, these being useless for many of the forms to be presently described. With this method the macronucleus of the Trypanosoma is stained a clear, transparent, crim-

son lake, the micronucleus a deep red, and the protoplasm a delicate blue; these reactions are constant throughout all the stages of its life-history.

The protoplasm of the adult *Trypanosoma* does not stain uniformly, as does that of some of the other forms, but there are parts faintly stained and parts unstained which is again in favour of the alveolar structure mentioned above. The vacuole is quite distinct as a clear round space, when the organism is stained by this method.

The macronucleus is generally of an oval or elongated shape, and it may be either uniform in colour, or in the form of fine threads; this latter is seen especially in those forms which show other signs of division. The micronucleus is seen as an intensely stained round dot, or as a short rod, this latter form again being seen in those forms which show other signs of approaching division. With the highest powers (1·5 apochromatic objective and 18 compensating eyepiece of Zeiss) we have not been able to make out any special structural characters in this body. The flagellum is not stained by this method, but if the preparation has been well fixed, it is easily visible; the vibratile membrane also is unstained, and can be generally better studied in specimens stained by simple stains, preferably thionin.

As regards the movements of the organism, in preparations where no pressure is exercised, they can be seen moving either with the flagellum or with the blunt end in front; but we think that the commoner mode of progression is with the flagellum forward.

The size and length of the body varies very much with the period of the disease at which the blood is examined, and with the kind of animal. The largest forms we have seen have been in rat's blood, just after death, and the smallest in rabbit's blood, early in the disease.

B. *Distribution of the Trypanosoma.*

1. *In the body of Normal Animals.*

(a) *In the Blood.*—We have found the flagellate form in the greatest numbers in the blood of the mouse, towards the end of the disease. In the rat also they occur in great numbers, and in both these animals they can be found in the blood on the fourth or fifth day. In the dog large numbers can be seen in the blood from the sixth day. In the cat they are fewer in number in the same lapse of time than in any of the animals before mentioned.

The rabbit seems to be the most refractory animal of any we have as yet used, and the *Trypanosoma* are found in the blood in small numbers only, and at very uncertain intervals.

(b) *In the Lymphatic Glands.*—In the superficial glands nearest to the point of inoculation the flagellate organism can be found earliest. In the rat the *Trypanosoma* can be found in the nearest superficial gland

in twenty-four hours after inoculation. We have not found that generalisation of the organism in the lymphatic glands occurs until nearly the end of the disease, when the organism is present in very large numbers in the blood. In the rabbit, in which the organisms are few or rare in the blood, the glands do not show any marked change, and the Trypanosoma are not readily found in them. Many other forms are found in the glands, to which reference will be made below.

(c) *In the Spleen.*—The adult Trypanosoma is found in but small numbers in the spleens of the various animals we have examined; but other forms are found there which will be described later. The enlargement of the spleen is *post mortem* the most obvious fact in the morbid anatomy of the disease; it may attain even to four or five times the average volume—this is especially the case in the rat.

(d) *In the Bone-marrow.*—We have found either very few flagellate organisms or none at all, in the bone-marrow of the various animals we have worked with. The marrow is altered in colour and structure, but there does not seem to be a greater number of Trypanosoma than can be accounted for by the blood in the marrow.

In the other organs and parts the number of organisms present depends upon the relative quantity of blood in the part.

2. *In the Body of Spleenless Animals.*—As the spleen in the ordinary animals is the organ which is most obviously altered in this disease, we have made a series of inoculations into animals (dog, cat, and rabbit) from which the spleen had been removed a year ago. In the dog, the adult forms of the Trypanosoma are not found so early in the blood of spleenless as in that of ordinary animals (seventh day as compared with fourth day after inoculation). The glands, after death, are much more generally enlarged, and are reddish in colour, and contain many more organisms than in the normal animal. Both the blood and glands contain, however, numerous other forms to be described below.

This marked difference in the colour of the glands of spleenless animals is probably due to the removal of the spleen, and the glands consequently taking on some of the splenic functions.

The bone-marrow is much altered, and in it likewise are found a large number of Trypanosoma: both flagellate and what are termed below "amoeboid" forms.

In the cat the conditions of experiment were altered, the blood (1 c.c.) from the infected animal being introduced, with every precaution to avoid contamination of the tissues, direct into the jugular vein. In this case the organism appeared in the blood in numbers on the fourth day, and the animal died on the twelfth day. As the Trypanosoma were introduced into the blood stream direct, there was no marked glandular enlargement, but the glands were all reddish in colour, the

change in colour being due to the spleenectomy. A few adult organisms were found in the glands and in the bone-marrow.

In the spleenless rabbit a few Trypanosoma have been found in the blood on two occasions, but the animal lived nearly two months, and notwithstanding the failure to detect adult flagellate forms in the blood on numerous occasions, the blood was always infective, and contained numerous forms termed "ameeboid" and "plasmodial" below.

C. Infectivity.

(a) *In Ordinary Animals*.—The blood and organs of an animal dead of the disease lose, before twenty-four hours after death, their infective power. This is apparently due to the rapidity with which decomposition sets in after death, as we have found living Trypanosoma in film preparations, made as described above, as long as five to six days after removal of the blood from the body; and we have also found that large quantities (200 c.c.) of blood removed from the body into a sterile vessel and kept in an atmosphere of oxygen, retain their virulence for at least three days, notwithstanding the fact that the flagellate form cannot be demonstrated.

We have found that the blood of the dog is infective at least two days before any adult Trypanosoma can be seen in the blood; and we have also found that the blood of the spleenless rabbit, in which we have only on two occasions seen any adult forms, is invariably infective. This of course suggests the idea that the organisms must be present in another form, and we have been able, by the use of the method of staining described above, to demonstrate the presence of other forms in the blood and organs, and have shown, by the experiments just mentioned, that the infectivity of the blood, in cases where there are no flagellate forms discoverable, depends in all probability upon the presence of one of the other forms which the Trypanosoma assumes.

Although a differential staining method, such as the one we have used, is necessary for following and demonstrating the various stages of the life-history of the Trypanosoma, still these stages can be seen in unstained living specimens, with very careful illumination. As a matter of fact, our first observation of them was in unstained preparations.

In the blood of the dog, cat, rabbit, rat, and mouse, besides the adult forms as described above, which, as mentioned, are very various in size, there are adult forms undergoing division, both longitudinal, and transverse, to which reference will be made later. Also two organisms are sometimes seen with their micronuclei in close apposition, or fused together, with more or less of their bodies also merged together. Such forms we believe are conjugations. Again, there are other large forms, with or without a flagellum, in which the chro-

matin of the macronucleus is broken up into a number of tiny granules, not bigger often than the micronucleus. Besides these there are other forms, which we call for convenience here "amoeboid" forms, by which term we mean single, small, irregularly shaped forms, with or without a flagellum, but always with a macro- and micro-nucleus. These nuclear structures are generally surrounded by a very delicate envelope of protoplasm, of greater or lesser extent, but occasionally forms are seen which seem to consist only of chromatin, with or without a flagellum. Besides these, again, there are other forms which we call, also for convenience, "plasmodial" forms, meaning thereby an aggregation or fusion of two or more amoeboid forms. In the blood these plasmodia are not generally very large, but may show evidence of from two to eight separate elements. Signs of division are very common; but in the blood one does not often meet with a plasmodium dividing up into more than four organisms of the adult shape. The plasmodial form also retains intact the two nuclear structures—the macro- and micro-nucleus—which we believe divide in the plasmodium, thus increasing its size.

In the spleenless animals the blood may contain no forms but the amoeboid and plasmodial, such as is the case in the rabbit, yet this blood is infective; moreover, in the dog, before the adult organism appears in it, the blood is infective, and therein, at this period, these plasmodial forms can be demonstrated. In the glands these plasmodial forms are found, but only in quantity in those animals from which the spleen has been removed.

The spleen is the organ which shows these forms in the greatest abundance. The whole spleen is crammed in every part with plasmodia, which are wedged in between the splenic cells in every direction: many amoeboid forms, and also immature flagellate forms are also seen, but the most striking thing is the enormous quantity and uniform distribution of the plasmodia. The great enlargement of the spleen, which we have found constant in all the animals we have used, is caused by this mass of plasmodia, which we have found in the spleen within forty-eight hours from the time of inoculation.

In the marrow these plasmodial forms are only found, so far as our experience goes, in those animals from which the spleen has been removed. In these cases there are both plasmodial and amoeboid forms in the marrow, the latter the more abundant.

The principal differences in the distribution of the plasmodial forms in animals with and without spleens is this: that in the animals with spleens the organ of choice for the plasmodia is the spleen, but they are also found constantly in the blood, and in less quantity in the glands, whereas in animals from which the spleen has been removed the plasmodial forms are plentiful in the blood, the glands, and the bone-marrow.

D. *Life-History of the Trypanosoma "Brucii."*

Besides the forms mentioned above, we have seen in the blood and in the organs divisions of the adult form, both longitudinal and transverse, the former the more frequent; but we think that this direct mode of reproduction is far less common than the indirect by means of conjugation (probably), breaking up of chromatin, production of amoeboid forms, with subsequent division of these amoeboid forms, and the formation of plasmodia by the aggregation or fusion of the amoeboid forms, and these finally giving off flagellate forms, at first small, and gradually increasing up to the normal adult form.

So that we should tentatively summarise the life-history of the Trypanosoma found in Tsetse Fly disease, which we think might properly be called "*Trypanosoma Brucii*," in recognition of the work done in connection with it by its discoverer Major Bruce, F.R.S., as follows:—

1. Reproduction by division, this being of two kinds:—

- (a) Longitudinal, the commoner.
- (b) Transverse, less frequent.

2. Conjugation, consisting essentially, so far as our observations go, of fusion of the micronuclei of the conjugating organisms.

(a) After this we are inclined to place those forms mentioned above, in which the chromatin is broken up, and scattered more or less uniformly through the whole body of the Trypanosoma, since this occurs after conjugation in other organisms not far removed biologically from this one. The next stage in our opinion is the amoeboid; we think that the flagellate form becomes amoeboid perhaps after conjugation, but also probably apart from this process.

(b) Amoeboid forms. These are found with and without flagella, of various shapes and sizes, but always possessing a macro- and micro-nucleus. These forms are constantly seen in the process of division, and sometimes are very irregular in shape, with, in this case, an unequal number of macro- and micro-nuclei, the latter being the more abundant. The amoeboid forms then fuse, or aggregate, together to form—

(c) The plasmodial forms. Whether these are true plasmodia, or whether they are only aggregations of amoeboid forms it is not yet possible to say, but as many related organisms form true plasmodia we are inclined to look upon these masses, provisionally, as true plasmodia. In the spleen these plasmodia reach a large size. From these again are given off—

(d) Flagellate forms, which increase in size, and become the ordinary adult form. Small flagellate forms are not infrequently seen in process of separation from the margin of these plasmodial masses.

Besides these forms we have observed frequently, especially in rat's

blood after death, the adult forms arranged in clumps. They appear, upon watching them for a considerable time, to get tangled together to form a large writhing mass ; then the movements become gradually slower in the centre of the mass, and are only seen at the periphery. At this stage, if the specimen be fixed, the mass appears to be made up of a quantity of macro- and micro-nuclei, as the protoplasm does not stain, except in the organisms at the periphery, *i.e.*, those which have arrived latest. Eventually these, too, become motionless, and the mass becomes an indistinct collection of granular matter, which is not infective, so that we look upon these tangles as a proof of death.

Since these observations were made, there has been published an important paper on the Rat Trypanosoma, by Lydia Rabinowitsch and Walter Kempner in the 'Zeitschrift für Hygiene,' vol. 30, Part 2. We have been able to confirm many of the observations and statements as to the morphology and reproduction of the Trypanosoma made by these writers. But there is no mention made of the plasmodial stage, or of any reproductive stage elsewhere than in the blood ; and the writers recognize only three methods of reproduction, namely, longitudinal and transverse division, and division by segmentation. This segmentation, they consider, arises from *one* organism, and they state that it may divide up into as many as ten to sixteen elements. This segmentation form would seem to correspond to our plasmodial stage, but we have seen much larger masses than those mentioned above, and they do not notice the enormous masses of plasmodia which infiltrate the spleen in every direction, and which can be found also in glands, and marrow. Moreover, their amoeboid stage (Kugelform) would precede the segmentation form, and therefore the "Kugelform" should be much larger than the ordinary adult form, but we have observed that as a rule, our amoeboid forms are very much smaller than the adult forms, some not being visible with any but the highest magnifying powers ; so that we have been unable to accept this form of division by segmentation, except in the form in which we have described it above, *i.e.*, our plasmodial stage.